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Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Bruce A. Sullenger, Ph.D.

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27710

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13. ABSTRACT (Maximum 200 Words) This project explores the potential of a new and innovative approach to human gene therapy that may prove to be useful for the treatment or prevention of a range of genetic diseases including many types of cancer. We have previously demonstrated that a trans-splicing group I ribozyme can be employed to repair mutant transcripts in <i>E. coli</i> (Sullenger and Cech, <i>Nature</i> 1994) and mammalian cells (Jones et al., <i>Nature Medicine</i> 1996; Lan et al., <i>Science</i> 1998). Ribozyme-mediated repair of mutant mRNAs associated with a range of human diseases is now experimentally tractable, and we have begun to assess the therapeutic potential of this process for the repair of mutant transcripts implicated in the development and progression of breast cancer. Because mutation of the p53 gene is the most common genetic change seen in a wide variety of malignancies including breast cancer, we have initially focused on repair of mutant p53 transcripts. Toward this end, we have mapped the accessible sites for ribozyme binding on p53 RNAs, and using this information we have generated ribozymes that can react with and repair mutant p53 transcripts in breast cancer and other cell lines.				
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4. Introduction

The overall goal of this project is to develop a new and innovative approach to human gene therapy that is based upon ribozyme-mediated repair of mutant mRNAs (Sullenger and Cech, 1994; Sullenger and Cech, 1995; Sullenger, 1995). This approach may be especially useful for the treatment of cancer because it should restore the regulated expression of oncogenes. Previously, we described how a trans-splicing ribozyme can be employed to repair mutant mRNAs in *Escherichia coli* (Sullenger and Cech, 1994). Subsequently we demonstrated that trans-splicing can also proceed in mammalian cells (Jones et al., 1996). Finally, we recently demonstrated that ribozymes could be employed to repair sickle β -globin transcripts in erythrocyte precursors from patients with sickle cell disease (Lan et al., 1998) and mutant p53 RNAs in a variety of human cancer cell lines (Watanabe and Sullenger, 2000, Appendix).

Many mutant messenger RNAs from dominant and suppressor oncogenes have been identified that appear to be involved in tumorigenesis. Repair of any of these mRNAs may yield new insights into tumor development and treatment. However, we have initially focused upon the repair of mutant transcripts from the p53 tumor suppressor gene. Much evidence indicates that loss of the p53 protein is associated with neoplastic transformation. Mutant p53 transcripts are frequently found in a range of primary human tumors and tumor cell lines including mammary carcinomas (Nigro et al., 1989; Bartek et al., 1990; Malkin 1994). Thus we have sought to develop ribozymes that can repair the mutant p53 transcripts present in these tumor cells in hope of using such ribozymes to either revert the transformed phenotype of these cells or induce apoptosis. Toward this end the specific objectives of this research proposal have been:

- 1.) To determine which regions of mutant p53 transcripts are accessible to trans-splicing ribozymes.
- 2.) To construct trans-splicing ribozymes that can repair mutant p53 transcripts and test them in vitro.
- 3.) To evaluate ribozyme-mediated p53 repair in tissue culture cells after transient transfection.
- 4.) To determine if the ribozyme can repair endogenous mutant p53 transcripts in breast cancer cell lines harboring defective copies of the p53 gene.
- 5.) To ascertain if repair of mutant p53 transcripts renders breast cancer cells less tumorigenic.

5. Body

5.1. Overview

We made significant progress toward fulfilling several of our specific aims since we received this grant on August 15th, 1997. One accomplishment is that we have determined which regions of the p53 transcript are accessible to trans-splicing ribozymes. Such mapping has been performed upon p53 transcripts generated by in vitro transcription and mutant p53 mRNAs present in total cellular RNA isolated from MDA-MB-231 mammary carcinoma cells. These

results fulfill our first specific aim: to map mutant p53 transcripts. In addition, we constructed two trans-splicing group I ribozymes that can recognize two of the most accessible regions on the p53 mRNA and have tested the ability of these ribozymes to react with the intended nucleotide on the targeted p53 transcript. Our results demonstrate that such trans-splicing ribozymes can react with p53 transcripts with high fidelity in the test tube as well as in MDA-MB-231 breast cancer cells. More recently, we have constructed plasmids containing ribozyme expression cassettes and have been able to demonstrate that trans-splicing ribozymes can repair mutant p53 transcripts in a variety of transiently transfected human cancer cell lines. These results fulfill our specific aims #2, #3 and 4: to create trans-splicing ribozymes that can repair p53 transcripts and test their activity in test tubes and in cells.

5.2 Studies and Results on Objective #1: To determine which regions of mutant p53 transcripts are accessible to trans-splicing ribozymes (six months).

To ascertain which regions of the p53 transcript are accessible to ribozymes, we developed an RNA mapping strategy that is based on a trans-splicing ribozyme library and RNA tagging (Jones et al., 1996). To generate the mapping library, the guide sequence of the *Tetrahymena* group I trans-splicing ribozyme was randomized such that the 5' end of the RNAs in the library began with 5'-GNNNNN-3' where "G" represents guanine and "N" represents equal amounts of the 4 nucleotides. To map the p53 transcript in vitro, the mapping library was incubated under splicing conditions with either p53 transcripts generated by in vitro transcription using T7 RNA polymerase or with p53 transcripts present in total cellular RNA isolated from MDA-MB-231 breast cancer cells. To identify accessible uridine residues, the trans-splicing reaction products were reverse transcribed (RT) and amplified by the polymerase chain reaction (PCR) with primers specific for the ribozyme's 3' exon tag (Jones et al., 1996) and for the 5' end of the p53 target RNA. From this analysis, the uridines at positions 41, 65 and 384 of p53 RNA appeared to be particularly accessible in p53 transcripts regardless of whether the p53 transcripts were generated by in vitro transcription or isolated from MDA-MB-231 mammary carcinoma cells (Watanabe and Sullenger, 2000, Appendix)). These mapping results, taken together with the fact that tumor cells often have mutations downstream of the nucleotide at position 360, encouraged us to focus on developing ribozymes that recognize the uridines present at positions 41 and 65 on the p53 mRNA. Thus, the internal guide sequence on the L-21 trans-splicing ribozyme was changed to 5'-GGAGGG-3' to generate a ribozyme, called Rib41, specific for site 41 and to 5'-GGGUCU-3' to generate a ribozyme, called Rib65, specific for site 65. In addition, inactive versions of these ribozymes, called Rib41d and Rib65d, which lack part of the catalytic core of the enzyme, were generated as controls (Sullenger and Cech, 1994).

5.3 Studies and Results on Objective #2: To construct trans-splicing ribozymes that can repair mutant p53 transcripts and test them in vitro (six months).

Rib41 and Rib65 can trans-splice a 3' exon tag onto p53 transcripts in vitro. The trans-splicing ribozymes, Rib41-3'tag and Rib65-3'tag, were incubated under splicing conditions with p53 RNA generated by in vitro transcription or total RNA isolated from MDA-MB-231 cells. To determine if trans-splicing had

occurred in any of the RNA samples, RT-PCR analyses were performed with one primer specific for the p53 target RNA and the other primer specific for the 3' exon tag sequence. An amplified fragment of the expected size was generated from samples containing Rib41-3'tag and Rib65-3' Tag and either in vitro transcribed p53 RNA or total RNA isolated from breast cancer cells (unpublished results). No such RT-PCR product was generated from samples that lacked a ribozyme or that contained the inactive versions of the ribozymes. Sequence analysis of the spliced products demonstrated that the ribozyme had reacted with the intended uridine. Subsequently, we changed the 3'-exon attached to Rib41 so that it encodes the wild type sequence for the p53 transcript. Using this ribozyme, we have been able to demonstrate that this ribozyme can repair p53 transcripts, that contain a point mutation at nucleotide 820, in the test tube (unpublished results).

5.4. Studies and Results on Objective #4: To determine if the ribozyme can repair endogenous mutant p53 transcripts in breast cancer cell lines harboring defective copies of the p53 gene (nine months).

To determine if Rib41-3'tag could react with mutant p53 transcripts inside human mammary carcinoma cells, the ribozyme was introduced into MBA-MB-231 cells via liposome-mediated transfection. RT/PCR amplification generated a fragment of the expected size (87 base pairs) from the total RNA isolated from the MBA-MB-231 cells that had been transfected with the active ribozyme (Watanabe and Sullenger, 2000; Appendix). By contrast no such product was generated from RNA samples isolated from cells that were not transfected or were transfected with the inactive ribozyme, Rib41d-3'tag. When Rib41-3'tag was added to the RNA extraction buffer used to isolate total RNA from a sample of mock transfected MBA-MB-231 cells, no amplification product was generated suggesting that the observed trans-splicing products were generated inside the transfected cells and not during RNA analysis. From these results we conclude that Rib41 can react with p53 transcripts inside human breast cancer cells. To confirm this result, we subcloned and sequenced a number of these PCR generated cDNAs. The splice junctions on each of the 6 clones tested were identical. This sequence analysis demonstrated that the ribozyme had reacted with the uridine at position 41 in the p53 transcript inside of these cells with high fidelity because in all 6 clones sequenced the 3' exon tag had been attached onto the proper uridine (Watanabe and Sullenger, unpublished results).

5.5. Studies and Results on Objective #3: To evaluate ribozyme-mediated p53 repair in tissue culture cells after transient transfection. (nine months).

To determine if Rib41 and Rib65 could repair mutant p53 transcripts following transient transfection, we first cloned the ribozymes into expression cassettes containing the CMV immediate early promoter and the preproinsulin polyadenylation signal sequence. A number of expression cassettes were constructed that contained a variety of guide sequence lengths as well as the presence or absence of the P10 region of the ribozyme. Each of these ribozyme constructs were individually cotransfected with an expression cassette containing a truncated version of the p53 gene into Saos-2 human osteosarcoma cells which do not naturally containing p53 derived transcripts. Total RNA was harvested from these cells and analyzed for the

presence of the repaired p53 transcript by reverse transcription and polymerase chain reaction techniques. Repaired RNAs could only be detected in RNA samples from cells expressing the active ribozymes and the truncated p53 substrate transcript; whereas, no repaired p53 RNA was detected in inactive ribozyme transfected cells (Watanabe and Sullenger, 2000; Appendix A). Moreover, we demonstrated that these repaired transcripts were translated to produce functional p53 protein in Saos-2, SW480 and Calu-6 cells (Watanabe and Sullenger, 2000; Appendix).

6. Key Research Accomplishments

- Mapped accessible regions for ribozyme binding on p53 transcript
- Demonstrated the trans-splicing ribozymes can react with mutant p53 transcripts in breast cancer cells.
- Demonstrated that RNA polymerase II expressed ribozymes can repair mutant p53 transcripts in Saos-2 cells.
- Demonstrated that repaired p53 transcripts are translated to produce functional p53 protein in a variety of cancerous cells.

7. Reportable Outcomes

Watanabe, T. and Sullenger, B.A. (2000). Induction of wild-type p53 activity in human cancer cells by ribozymes that repair mutant p53 transcripts. *Proc. Natl. Acad. Sci. USA* 97, 8490-8494.

8. Conclusions

In summary, we made significant progress toward fulfilling several of our specific aims since we received the funding for this research proposal on August 15th, 1997. We have determined which regions of the p53 transcript are accessible to trans-splicing ribozymes using a novel RNA mapping strategy based upon a library of ribozymes and 3'-exon tagging. Such mapping has suggested that the nucleotides at positions 41 and 65 on the p53 transcript are particularly accessible to trans-splicing ribozymes. These results fulfill our first specific aim: to map the accessible regions on mutant p53 transcripts. In addition, we constructed two trans-splicing group I ribozymes that can recognize the uridines at positions 41 and 65 on the p53 mRNA and have tested the ability of these ribozymes to react with the intended nucleotide on the targeted p53 transcripts in the test tube and inside the human mammary carcinoma cell line MDA-MB-231. Our results demonstrate that these trans-splicing ribozymes can react with p53 transcripts with high fidelity in the test tube and that Rib41 can react with mutant p53 transcripts in breast cancer cells. More recently, we have generated a number of ribozyme expression cassettes and have been able to demonstrate that the ribozyme can repair truncated p53 transcripts following expression in cotransfected Saos-2 osteosarcoma, SW480 colorectal carcinoma and Calu-6 lung adenocarcinoma cells. These results partially fulfill specific aims #2, 3 and 4: to create trans-splicing ribozymes that can repair p53 transcripts and test their activity in test tubes and in cells. These results demonstrate that trans-splicing ribozymes represent a novel means to induce the activity of tumor suppressor gene function in transformed human cells. Once adequate gene transfer methods have been developed this approach may prove useful for the treatment or prevention of human breast and other forms of cancer.

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10. Final Report Bibliography and Support Information

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Dr. Takashi Watanabe

Dr. Bruce A. Sullenger

Induction of wild-type p53 activity in human cancer cells by ribozymes that repair mutant p53 transcripts

Takashi Watanabe and Bruce A. Sullenger*

Center for Genetic and Cellular Therapies, Departments of Surgery and Genetics, Duke University Medical Center, Durham, NC 27710

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Several groups have attempted to develop gene therapy strategies to treat cancer via introduction of the wild-type (wt) p53 cDNA into cancer cells. Unfortunately, these approaches do not result in regulated expression of the p53 gene and do not reduce expression of the mutant p53 that is overexpressed in cancerous cells. These shortcomings may greatly limit the utility of this gene replacement approach. We describe an alternative strategy with *trans*-splicing ribozymes that can simultaneously reduce mutant p53 expression and restore wt p53 activity in various human cancers. The ribozyme accomplished such conversion by repairing defective p53 mRNAs with high fidelity and specificity. The corrected transcripts were translated to produce functional p53 that can transactivate p53-responsive promoters and down-modulate expression of the multidrug resistance (*MDR1*) gene promoter. The level of wt p53 activity generated was significant, resulting in a 23-fold induction of a p53-responsive promoter and a 3-fold reduction in *MDR1* promoter expression in transfected cancer cells. Once efficient delivery systems are developed, this strategy should prove useful for making human cancers more responsive to p53 activity and more sensitive to chemotherapeutic agents.

In many cancers, the p53 tumor suppressor gene is mutated, and the most frequent changes are missense point mutations (1) that cluster in the central region of the gene that encodes the DNA-binding domain of the p53 protein (2). p53 is a transcription factor (3, 4) that binds as a tetramer to specific DNA recognition sequences (5, 6) adjacent to p53-responsive genes that engender cell-cycle arrest (7) and apoptosis (8). The mutant p53s lose sequence-specific DNA-binding properties (2, 5, 6) and cannot regulate expression of those genes (3, 4, 7, 8). Moreover, by binding to and forming inactive tetramers with wild-type (wt) p53, certain mutant versions of the p53 protein can transform cells neoplastically, presumably by inhibiting endogenous wt p53 function in a dominant-negative fashion (9, 10). Finally, certain mutant p53 proteins have acquired a "gain-of-function," defined as the ability to augment cell proliferation in the absence of endogenous wt p53 (11) and resulting in phenotypic changes such as the overexpression of the multidrug resistance (*MDR1*) gene (11, 12), which can confer cross-resistance to cytotoxic drugs (13).

Several clinical trials that attempt to introduce expression cassettes for the wt p53 cDNA into cancer cells are underway to evaluate the safety and potential utility of this strategy to combat cancer (14). One significant limitation of this gene transfer approach is the inability to properly regulate gene expression after gene transfer. A second potential complication is that most p53 mutations found in human cancers are not null mutations but rather encode mutant versions of the p53 protein that may have unwanted activities such as a gain-of-function or be dominant negative inhibitors of wt p53 activity (11, 15). Therefore to treat these types of cancers with p53 therapy, one would ideally attempt to restore wt p53 production while simultaneously reducing or eliminating deleterious mutant p53 protein expression.

The self-splicing group I intron from *Tetrahymena thermophila* has been shown previously to mediate *trans* splicing of an exon attached to its 3' end onto a targeted 5' exon RNA that is a separate RNA molecule *in vitro* (16, 17). More recently, we demonstrated that a slightly shortened version of this ribozyme could repair truncated *lacZ* transcripts in *Escherichia coli* (18) and in cultured mammalian cells (19). In these experiments, the ribozyme recognized the *lacZ* substrate RNA by base pairing to it through the internal guide sequence (IGS) of the ribozyme. Target RNAs can be recognized by the ribozyme at any accessible uridine residue found upstream of the mutation(s) in the target transcript that is to be corrected. The ribozyme then cleaves the target RNA, releases the downstream RNA sequence containing the mutation(s), and replaces the sequence with a 3' exon that encodes the correct sequence for the wt transcript (Fig. 1). In the process, the regulated expression pattern of the endogenous gene should be maintained.

Two recent reports demonstrated that *trans*-splicing ribozymes can be used to repair clinically relevant mutant RNAs in two different human cell types (20, 21). However, neither report demonstrated that the repaired RNAs could be translated to generate functional protein. The question as to whether such *trans*-spliced transcripts can be translated in mammalian cells is especially important in light of recent work that demonstrated that transcripts do not seem to be translated efficiently after self splicing of a group I intron (22). In the studies described herein, we set out to determine whether *trans*-splicing group I ribozymes can be used to repair a variety of defective p53 transcripts and in the process restore p53 transcriptional activity to treated cells.

Materials and Methods

Mapping Accessible Sites on p53 RNA. The mapping library (GN5) was generated as described (20). The *Bam*HI fragment of a plasmid pC53-SN3 (a generous gift from B. Vogelstein, Johns Hopkins University School of Medicine, Baltimore) was inserted into the multiple cloning site of pBluescript SK (+) (Stratagene), and the p53 cDNA was transcribed by T7 RNA polymerase as a run-off transcript that contained the first 432 nt of the p53 mRNA. Transcription of ribozymes and the p53 RNA was performed under the same condition as described (23), except that 5 mM MgCl₂ and 25 mM MgCl₂ were used for the ribozyme and the substrate, respectively. The GN5 library (100 nM) and either p53 RNA (10 μ M) transcribed *in vitro* or cellular RNA (1–2 μ g) were denatured at 95°C for 1 min and then preequilibrated.

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Abbreviations: wt, wild-type; CMV, cytomegalovirus; IGS, internal guide sequence; RT, reverse transcription.

*To whom reprint requests should be addressed. E-mail: b.sullenger@cgct.duke.edu.

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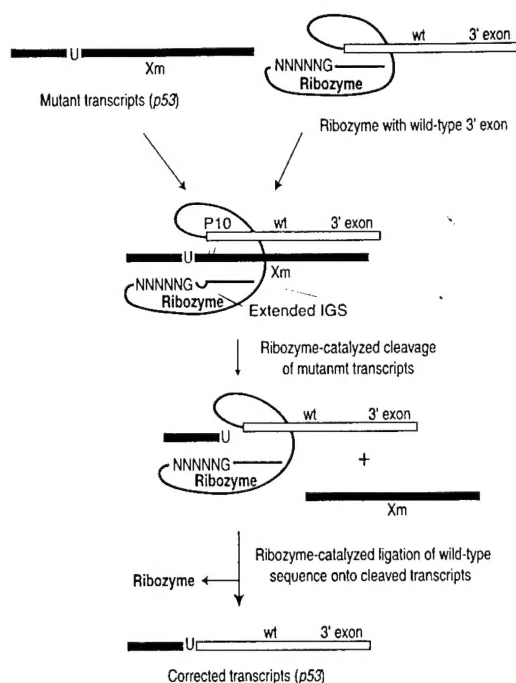


Fig. 1. Schema to correct mutant *p53* transcripts with targeted *trans* splicing. Mutant messages can be recognized by a ribozyme at any uridine upstream of the mutation (marked by Xm) by base pairing to the sequence through its IGS. The ribozyme then removes the mutation-containing sequence and replaces it with a 3' exon that encodes the correct sequence for the wt transcript. The extended IGS and P10 interactions are shown.

brated in the reaction buffer (50 mM Hepes, pH 7.0/150 mM NaCl/5 mM $MgCl_2$) at 37°C for 2 min. The substrates were then added to the ribozymes along with guanosine (100 μ M) to start the *trans*-splicing reactions, which proceeded at 37°C for 4 h. The *trans*-splicing reaction products were reverse transcribed and amplified by PCR as described (19).

Construction of Ribozyme Expression Vectors. To generate Rib41-3' p53 and Rib65-3' p53, PCR was performed with *Pfu* polymerase (Stratagene) and with pC53-SN3 as a template. The PCR products containing 1,141 nt of *p53* 3' exon for Rib41 and 1,117 nt for Rib65 were digested with *Bam*HI and inserted between the *Nru*I and *Bam*HI sites of pL-21 multiple cloning site or pL-21 multiple cloning site dead (a catalytically inactive version of the intron described previously; ref. 18). Each antisense region of the ribozyme was synthesized by Klenow fragment (3' \rightarrow 5' *exo*⁻; New England Biolabs) after annealing of the two synthetic oligonucleotides to create a double-stranded DNA sequence that contains the 35- and 36-nt sequence for the antisense region and the 13- and 14-nt helix P1 of Rib41-3' p53 and Rib65-3' p53, respectively. After filling in, the products were digested by *Sac*I and *Sph*I and cloned into those sites of a pCS4R61g (provided by N. Lan, Duke University, Durham, NC) that harbor the cytomegalovirus immediate-early (CMV-IE) gene promoter and a polyadenylation signal of simian virus 40 virus to generate pRib41p53 and pRib65p53. The sequences of the PCR primers and the synthetic oligonucleotides used are available on request.

Repair of Truncated *p53* Transcripts in Human Osteosarcoma Cells. Saos-2 human osteosarcoma cells were seeded in six-well plates at a density of 2.0×10^5 cells per well 20 h before transfection. A truncated 5' *p53* expression plasmid, pC53 5' trunc, was constructed by deleting a *Pvu*II to *Sma*I fragment of pC53-SN3 (24). The cells were cotransfected with pRib41p53 or

pRib41p53 (5 μ g) with or without pC53 5' trunc (5 μ g) and 10 μ g of DMRIE-C (GIBCO/BRL). In the control experiments, the empty parental expression vector was substituted for either *p53* or ribozyme expression plasmid. Total RNA was isolated from cells 20 h after transfection by TRIzol (GIBCO/BRL). EDTA (45 mM) was added to TRIzol before RNA extraction to chelate magnesium ions and to inhibit ribozyme folding and catalysis. After the cells transfected with pC53 5' trunc alone and the cells transfected with pRib41p53 alone were solubilized in TRIzol, these two lysates were mixed in the "mix" control sample. RNA was reverse transcribed with a primer specific for the *p53* 3' exon sequence at 37°C for 15 min in the presence of 50 mM L-argininamide, a competitive inhibitor of ribozyme activity to squelch *trans* splicing during reverse transcription (RT). The resulting cDNAs were amplified for 40 cycles by a 5' primer specific for the *trans*-splicing junction (5'-GGGGG-GATCCGTCGAGCCCCATATCTCC-3'), including two mutations of C to A in a portion of 5' *p53* sequence to avoid amplification of Rib41-3' *p53* itself) and by a 3' primer specific for the *p53* cDNA sequence downstream of the splice junction.

Luciferase Assays on Extracts from Osteosarcoma, Colon Cancer, and Lung Cancer Cells. Saos-2 and SW480 human colorectal carcinoma (or Calu-6 human lung adenocarcinoma) cells were seeded in 100-mm plates at a density of 7.5×10^5 and 1.5×10^6 cells per plate, respectively, 18–24 h before transfection. The cells were cotransfected with PG13-luc (2 μ g; containing 13 copies of a *p53* DNA-binding consensus sequence linked to a luciferase reporter gene, a generous gift of B. Vogelstein) and a β -galactosidase expression vector pCMV β gal (1 μ g) along with pC53 5' trunc (5 μ g) and pRib41p53 (5 μ g) or pRib65p53 (5 μ g) with DMRIE-C (26 μ g) for Saos-2 cells and pRib41p53 (5 μ g) or pRib65p53 (5 μ g) along with DMRIE-C (16 μ g) for SW480 cells. As a negative control, the empty parental expression vector was substituted for either *p53* or ribozyme expression plasmid. In contrast to the active ribozyme constructs, transfection of the inactive ribozyme expression plasmids alone into Saos-2 cells resulted in a modest (2- to 10-fold) increase in luciferase activity, suggesting that the *p53*-derived 3' exons after the deleted introns were being translated, thus ruining their utility as controls in these experiments. In previous experiments with a 3' exon encoding the green fluorescent protein, we observed that several potential translational start sites exist within the sequence of the *Tetrahymena* group I intron that are activated by deletion of the ribozyme's core in the inactive ribozyme construct (unpublished results). Calu-6 cells were cotransfected with pMDR-luc containing the human *MDR1* promoter upstream of the luciferase gene (graciously provided by E. J. Stanbridge, University of California, Irvine; 3 μ g) and pCMV β gal (1 μ g) along with pRib41p53 (5 μ g) or pRib65p53 (5 μ g) with DMRIE-C (18 μ g). Cell lysates were harvested 24 h after transfection for Saos-2 cells and 48 h after transfection for SW480 and Calu-6 cells with the Reporter lysis buffer provided with the Luciferase Assay System (Promega) and assayed for luciferase activity by using a Lumat LB9507 (Berthold, Nashua, NH). Transfection efficiency was quantified by measuring β -galactosidase activity in the extracts, and luciferase values were normalized to account for differences in transfection efficiency.

Results

To ascertain which uridines in the *p53* transcript are accessible, we developed an RNA mapping strategy based on a *trans*-splicing ribozyme library (25) and RNA tagging (19). A ribozyme library was constructed that contains a randomized IGS, 5'-GNNNNN-3' (GN5), where G represents guanine and N represents equal amounts of the four nucleotides (Fig. 1). The library was incubated under splicing conditions either with a portion of *p53* RNA generated by *in vitro* transcription (nucle-

Table 1. *In vitro* mapping results of the ribozyme-accessible sites in the *p53* RNA

Reaction sites, nucleotide position*	Number of clones†	
	<i>p53</i> RNA‡ (<i>in vitro</i> transcribed)	Cellular RNA from MDA-MB-231 cells§
+41	4	4
+65	2	2
+307	—	1
+332¶	—	1
+340¶	1	1
+384¶	6	5
+389¶	—	1

*Nucleotide positions are indicated as the nucleotide numbers after the translation initiation site and presented for the accessible uridines identified from the mapping analyses.

†The number of individual clones containing a given uridine at the splice.

‡A portion of *p53* target RNA (nucleotides 1–432).

§A human breast cancer cell line that overexpresses a mutant *p53* mRNA (AGA to AAA at codon 280).

¶The *p53* gene is frequently mutated in human tumors downstream of nucleotide 328 (1).

otides 1–432) or with total RNA isolated from MDA-MB-231 human breast cancer cells, which overexpress a mutant version of *p53* mRNA (mutation at codon 280, AGA changed to AAA). The *trans*-splicing reaction products were amplified by RT-PCR with primers specific for the ribozyme's 3' exon tag (3' *lacZ* exon sequence) and the target *p53* RNA. Sequence analyses of the splice junctions showed that several uridines appeared accessible (Table 1). To delineate further which uridines are the most accessible, we evaluated a number of the ribozymes for their ability to cleave *p53* transcripts *in vitro*. Only ribozymes Rib41 and Rib65, which recognize the uridines at position 41 (U₄₁) and 65 (U₆₅) of the *p53* coding sequence, cleaved the majority of the *p53* target RNA to yield products of the expected size (data not shown). Moreover, *p53* RNA is most often mutated in human cancers at sites downstream of nucleotide 328 (1). Both reasons encouraged us to focus on further developing ribozymes Rib41 and Rib65.

To create Rib41 and Rib65 derivatives that could convert mutant *p53* transcripts into wt *p53* RNAs, we attached the wt human *p53* sequences to the ribozymes (nucleotides 42 through 1,179 for Rib41 and nucleotides 66 through 1,179 for Rib65) as a 3' exon. These ribozymes, called Rib41–3' *p53* and Rib65–3' *p53*, were expressed by using an RNA polymerase II-based transcription unit containing the CMV-IE gene promoter. Because group I *trans*-splicing ribozymes that contain 6-nt-long IGS are not very active after expression in bacteria (26), we extended the IGS of Rib41–3' *p53* and Rib65–3' *p53* such that they would contain an extended P1 and a 35-nt-long antisense region as well as a P10 helix. Moreover, we have been unable to detect *trans*-splicing activity after pol II-based expression of *trans*-splicing ribozymes that contain only 6-nt-long guide sequences in mammalian cells (N. Lan and B.A.S., unpublished work). To distinguish repaired *p53* transcripts from mutant *p53* RNA easily, we took advantage of the degeneracy of the genetic code to alter the sequence of the restorative *p53* 3' exon without changing the encoded protein. These changes also allowed us to amplify *trans*-splicing products selectively by using a 5' primer complementary to the cDNA derived from repaired *p53* RNAs and allowed us to reduce potential problems engendered by making the antisense region complementary to the 3' exon.

To test whether Rib41–3' *p53* could repair truncated *p53* transcripts in cancer cells, we cotransfected a plasmid containing the Rib41–3' *p53* expression cassette (pRib41p53) along with a plasmid containing the CMV promoter and a truncated *p53*

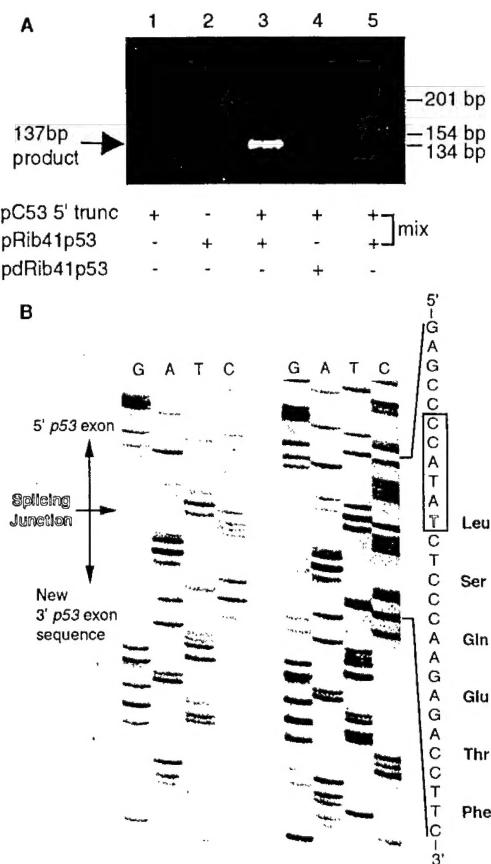


Fig. 2. (A) Repaired *p53* RNAs generated in Saos-2 cells. Cells were transfected with a truncated *p53* expression plasmid (pC53 5' trunc; lanes 1 and 3–5) alone (lane 1) or with the active (pRib41p53, lanes 2 and 3; lane 5 mix sample) or with the inactive (pdRib41p53, lane 4) ribozyme expression plasmid. Corrected *p53* RNAs were amplified by RT-PCR, yielding a DNA fragment of 137 bp. The migration of size markers of 134, 154, and 201 bp is indicated. (B) Sequences of amended *p53* transcripts produced in Saos-2 cells. Sequences of two representative clones are shown. The expected sequence for a corrected transcript around the splicing junction is shown along with the predicted translation product. The ribozyme recognition sequence is boxed, and the nucleotide at the position 41 is outlined.

cDNA (pC53 5' trunc) into the human osteosarcoma cell line, Saos-2, which does not express endogenous *p53* transcripts (27). Total RNA was isolated from transfected cells and analyzed by RT-PCR with primers specific to the repaired *p53* transcript. *Trans*-spliced products were detected only in RNA from cells transfected with pRib41p53 and pC53 5' trunc (Fig. 2A, lane 3). No such product was detected in cells transfected with pC53 5' trunc alone or in those cotransfected with pC53 5' trunc and an inactive ribozyme plasmid (pdRib41p53) that lacks part of the catalytic core of the enzyme (Fig. 2A, lanes 1 and 4). Moreover, no *trans*-splicing product was detected in a "mix" RNA sample, where lysate from pRib41p53-transfected cells was mixed with lysate from pC53 5' trunc-transfected cells before RNA extraction (Fig. 2A, lane 5). Sequence analysis demonstrated that the ribozyme had correctly repaired the truncated *p53* transcript by splicing its restorative *p53* 3' exon onto the U₄₁ of the mutant *p53* mRNA (Fig. 2B). Thus, this experiment indicated that the ribozyme was able to repair truncated *p53* transcripts in these human osteosarcoma cells and restore the ORF for translation of the *p53* protein inside these cells.

To determine whether the repaired *p53* transcripts were being translated to produce functional *p53* protein inside transfected

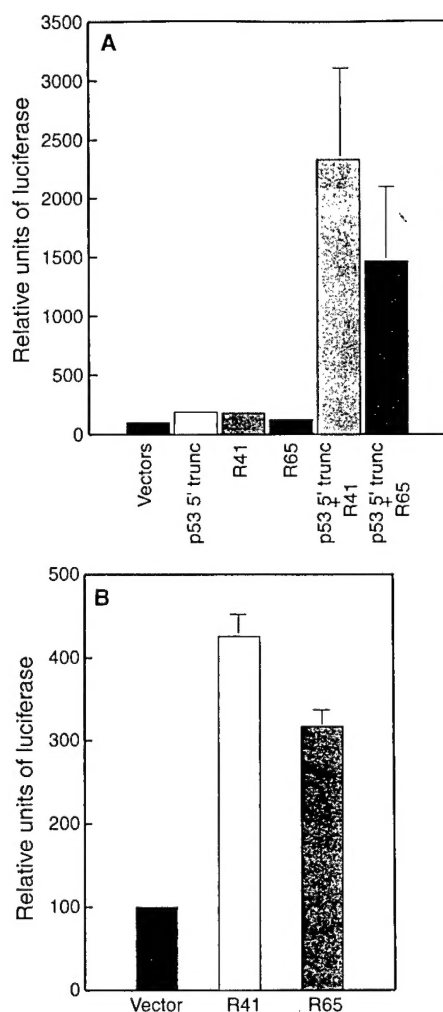


Fig. 3. p53-mediated transcriptional transactivation after RNA repair. (A) Saos-2 cells were transfected with the PG13-luc reporter construct and the empty parental expression vectors (Vectors), the truncated 5' p53 expression plasmid alone (pC53 5' trunc), each ribozyme expression plasmid alone [pRib41p53 (R41) or pRib65p53 (R65)], or pC53 5' trunc along with pRib41p53 or pRib65p53. (B) SW480 cells were transfected with the PG13-luc and each ribozyme expression vector (pRib41p53 and pRib65p53) or an empty vector. Relative luciferase activity was quantitated as a percentage of the vector control sample, and average values \pm SD for luciferase activity from three independent experiments are shown.

cells, we cotransfected a reporter plasmid containing the luciferase gene under the control of a p53-dependent promoter (PG13-luc) into the Saos-2 cells that had been transfected with the ribozyme and p53 substrate expression plasmids. Production of functional p53 was then monitored by measuring the induction of luciferase activity. In Saos-2 cells transfected with PG13-luc and the empty parental expression vectors, for both truncated p53 and ribozyme, only very low levels of luciferase activity were induced, as would be expected because Saos-2 cells do not produce endogenous p53 protein (27). Transfection of pRib41p53 or pRib65p53 along with pC53 5' trunc and PG13-luc in Saos-2 cells resulted in a 23-fold and a 15-fold increase in p53-induced luciferase activity, respectively (Fig. 3A). No significant increase in luciferase activity was noted in cells transfected either with ribozyme or with the substrate plasmid alone (Fig. 3A). These results demonstrate that *trans*-splicing ribozymes can repair mutant p53 transcripts to generate mRNAs that are translated to produce functional p53 protein. These

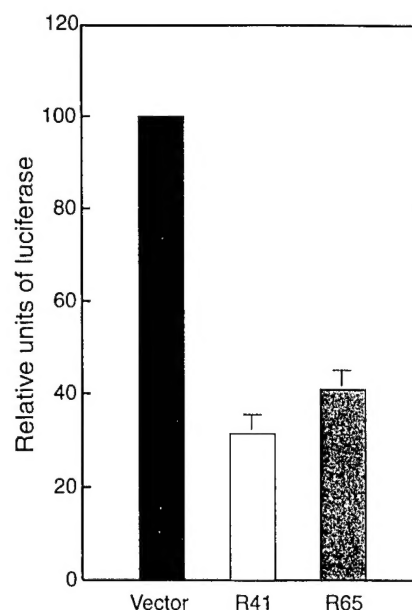


Fig. 4. Repression of transcription from the *MDR1* gene promoter after p53 repair. Calu-6 cells were cotransfected with the *MDR*-luc reporter construct and an empty vector or each ribozyme expression vector (pRib41p53 or pRib65p53). Relative luciferase activity was quantitated as a percentage of the vector control sample, and average values \pm SD for luciferase activity from three independent experiments are shown.

newly generated transcription factors can then transactivate p53 responsive promoters in human cancer cells.

To determine whether *trans*-splicing ribozymes can repair endogenous transcripts expressed from mutant p53 genes present in cancer cells and restore p53 function therein, we evaluated the ribozymes in the human colorectal carcinoma cell line SW480 that expresses a p53 gene harboring two point mutations (G₈₁₈ to A₈₁₈ and C₉₂₅ to U₉₂₅). Transfection of these cells with PG13-luc and a control vector resulted in the generation of only low levels of luciferase activity, because the cells express only mutant p53 protein that poorly transactivates luciferase gene expression (Fig. 3B). However, cotransfection of SW480 cells with PG13-luc and either pRib41p53 or pRib65p53 resulted in a 4-fold or 3-fold induction, respectively, of luciferase expression in these cells (Fig. 3B). By comparison, cotransfection of these SW480 cells with PG13-luc and pC53-SN3, which harbors the CMV-IE promoter and the wt p53 cDNA, stimulated luciferase production by approximately 180-fold (data not shown). Thus, the ribozymes are able to repair endogenous mutant p53 transcripts in human colorectal carcinoma cells, and the amended RNAs can be translated to produce functional p53 protein. Moreover, the amount of p53-mediated transcriptional transactivation generated after such repair can reach levels that are at least 1–2% of the level of p53 activity generated after high-level expression of the protein in these same cells with a strong, virally derived promoter.

Because it has been observed that certain mutant p53s activate transcription of the human *MDR1* gene, whereas wt p53 represses such expression (12), we sought to determine whether the wt p53 generated after RNA repair was able to repress mutant p53-mediated transactivation of the *MDR1* promoter in cancer cells. A construct containing the human *MDR1* promoter upstream of the luciferase gene (p*MDR*-luc) was cotransfected with pRib41p53 or pRib65p53 into the Calu-6 lung carcinoma cells that harbor a homozygous nonsense p53 mutation at codon 196 (CGA to TGA). Cotransfection of either pRib41p53 or pRib65p53 along with p*MDR*-luc significantly repressed tran-

scription from the *MDR1* promoter ($67 \pm 5\%$ and $60 \pm 6\%$, respectively; Fig. 4) as compared with the level of expression observed when the cells were cotransfected with p*MDR*-luc and a control vector (Fig. 4). By comparison, transfection of Calu-6 cells with pC53-SN3 suppressed activation of the *MDR1* promoter by approximately 95% (data not shown). Thus, the functional p53 protein generated after RNA repair can significantly repress the activation of the *MDR1* gene promoter by mutant p53 protein present in the lung adenocarcinoma cells. Moreover, this level of repression is approximately 65–75% the level obtained when high levels of p53 protein are expressed in the Calu-6 cells.

Discussion

We have used a *trans*-splicing group I ribozyme to amend mutant *p53* transcripts in human cancer cells (Fig. 1). To determine which regions of the *p53* transcript are accessible for ribozyme binding, we mapped the *p53* RNA with a *trans*-splicing ribozyme library (Table 1). Such analysis suggested that the uridines at positions 41 and 65 in the *p53* coding sequence are particularly accessible for ribozyme binding and activity. Using ribozymes designed to target these particular residues, we demonstrated that a single *trans*-splicing ribozyme can be used to revise a variety of different mutant *p53* transcripts in cells from different types of human cancers (Figs. 2–4). The revision of *p53* transcripts seems to proceed with high fidelity in these settings (Fig. 2). Moreover, we showed that the RNAs repaired by *trans* splicing go on to be translated to produce functional protein inside human cancer cells, which can lead to transactivation of a *p53* responsive promoter (Fig. 3 *A* and *B*) and down-modulate expression from the *MDR1* gene promoter (Fig. 4).

These results suggest that repair of defective *p53* RNA may become a useful strategy to engender production of functional *p53* activity in neoplastic cells to combat cancer. Aberrant expression of *p53* by conventional gene replacement strategies may lead to unintended phenotypic changes in both tumorigenic and normal cells (28, 29). For example, it has been demonstrated that overexpression of the wt *p53* gene aberrantly decreases growth rate and alters morphological differentiation of normal human keratinocytes (28). Thus, coordinated expression of the *p53* gene is apparently critical for proper growth, development, and differentiation of certain primary human cells, and incorrect expression can lead to dramatic phenotypic aberrations. By contrast, mRNA repair should result in the production of correct gene products at the appropriate times in human development and cellular differentiation, because the ribozyme can amend the

mutant mRNA only when it is expressed. Our observation that *p53* activity is engendered only in Saos-2 cells when such cells are cotransfected with a ribozyme plasmid and a plasmid encoding a truncated *p53* transcription unit highlights the fact that mRNA repair can occur only when substrate RNAs are present in the cell with the ribozyme (Fig. 3A). In addition, *trans*-splicing ribozymes can simultaneously reduce the production of a deleterious protein and induce the production of a functional gene product to lead to desired phenotypic changes in treated cells, as is the case when functional *p53* protein suppressed mutant *p53* protein-mediated activation of the *MDR1* promoter in Calu-6 cells after RNA repair (Fig. 4). Thus, mRNA repair represents a potentially very powerful approach to genetic therapy that offers gene therapists some unique advantages over currently used techniques.

Our findings that significant levels of *p53* activity can be generated from repaired transcripts are quite encouraging with regard to long-term development of this technology, especially because the efficiency of mutant *p53* RNA repair will almost assuredly not have to be 100% to benefit patients. The wt *p53* protein has been shown to be able to suppress cell growth totally and decrease colony formation when present at quantities 10-fold lower than mutant *p53* in neoplastic cells (30). Thus, repair of as little as 10% of the mutant *p53* transcripts may be efficient enough to revert neoplastic transformation, induce apoptosis, or render cancerous cells sensitive to lower doses of chemotherapeutic agents. To determine whether *trans*-splicing ribozymes can repair this level of *p53* RNA in neoplastic cells and impact the development and progression of cancer in animals, more efficient ribozyme delivery systems must be developed. Of the currently available gene delivery systems, adenoviral vectors may prove the most useful for such studies because of their ability to transfer genes fairly efficiently into a variety of human cancer cells (14). However, in the long term, new, less immunogenic, and even more efficient gene transfer systems will likely be required to deliver *trans*-splicing ribozyme in a clinically relevant fraction of premalignant or transformed cells. The demonstration that *trans*-splicing ribozymes can repair a variety of mutant *p53* transcripts and induce *p53* activity in a number of different human cancer cell lines should continue to encourage the development of more effective gene transfer systems.

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